Articles

NMR Studies of ^1H NOEs in Glycogen†

Wei Chen,*,‡ Malcolm J. Avison,§ Xiao-Hong Zhu,‡ and Robert G. Shulman‡

Department of Molecular Biophysics and Biochemistry and Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Received May 6, 1993; Revised Manuscript Received August 16, 1993®

ABSTRACT: We have examined the cross-relaxation behavior among the protons of oyster glycogen using nuclear Overhauser enhancement (NOE). Steady-state and transient NOEs were generated using low-power CW irradiation and frequency-selective inversions. In D_2O, saturation of glycogen H2 and H4′ at 3.64 ppm gave a strong negative NOE ($\eta = -0.74$) at H1. The NOE was similar to the value predicted by the correlation time (τ_c) calculated from the T_1 and T_2 of glycogen H1 in D_2O assuming an isotropic rigid motor dipole–dipole model. Selective inversion of H2 and H4′ gave a transient NOE at H1. In D_2O, selective inversion of H1 also led to negative transient NOEs in the H2+H4′, H3, and H5 resonances. The magnitude and rates of appearance of the NOEs in H3 and H5 were too large to arise from direct H1–H3 and H1–H5 dipolar interactions, but were consistent with very efficient cross-relaxation leading to large second-order NOEs. The glycogen H1 NOE in H_2O was also studied. Replacement of D_2O with H_2O as solvent significantly reduced the steady-state NOE at H1 following saturation of H2+H4′. Saturation of the water resonance caused a large negative NOE at H1 ($\eta = -0.55$) consistent with our earlier study which indicated that there was no direct dipolar interaction between H1 and free H_2O.

Glycogen is the main carbohydrate storage molecule in animals and man. The primary structure consists of α-1,4-linked glucose chains containing 12–13 glucose residues, with α-1,6 branched points (Gunja-Smith et al., 1970). Despite a molecular weight of up to 10^8, the ^{13}C and ^1H resonances of glycogen are relatively sharp *in vitro*, indicating a high degree of internal mobility (Sillerud & Shulman, 1983; Zang et al., 1990a,b, 1991). Studies of the relaxation properties of $^{13}C1$ and $^1H1$ of glycogen confirm this: Zang *et al.* found that the $^{13}C1$ T_1 of extracted rabbit liver glycogen can be described by a rigid rotor dipolar model with $\tau_c = 6$ ns or by a modified Lipari–Szabo model which gave $\tau_c = 3.9$ ns (Zang et al., 1990b), while Chen *et al.* have recently shown that the H1 relaxation can be described by a rigid rotor dipolar model with $\tau_c = 2.7$ ns (Chen et al., 1993). The original observation by Sillerud and Shulman gave an average value of $\tau_c = 4.6$ ns (Sillerud & Shulman, 1983). These studies indicated that the correlation times which dominate the $^{13}C1$ and $^1H1$ dipolar interactions are much shorter than the molecular rotational correlation time.

Our recent study of the ^1H relaxation properties of glycogen in D_2O showed that there is significant dipolar cross-relaxation between H1 and the intraring H2 and interring H4′ protons with $\tau_c = 2.7$ ns, assuming a rigid rotor dipolar model (Chen et al., 1993). The study also found that the longitudinal relaxation of H1 is enhanced in H_2O compared with D_2O. To further investigate the physical nature of the interactions which determine H1 relaxation, and the role of H_2O in glycogen relaxation, we have measured the nuclear Overhauser enhancement (NOE) of glycogen H1 in D_2O and H_2O.

THEORY

For the case of two identical homonuclear spins I and S, and dipolar relaxation, the time dependency of spin I z magnetization, I_z, is (Chen et al., 1993; Noggle & Schirmer, 1971)

$$I_z(t) = I_0[1 + C_1e^{-(\rho+\sigma)t} + C_2e^{-(\rho-\sigma)t}] \quad (1)$$

in which I_0 is the equilibrium value of I_z and ρ and σ are relaxation rates. The constants C_1 and C_2 depend on the initial magnetizations of spin I and spin S. Thus, the time dependence of I_z following a perturbation of S_z depends on the nature of the perturbation. Two cases are of interest.

(a) *Saturation of S*:

$$I_z(t) = I_0[1 + (\sigma/\rho)(1 - e^{-\rho t})] \quad (2)$$

which for long t (i.e., steady-state saturation of spin S) gives

$$I_z(\infty) = I_0(1 + \sigma/\rho) = I_0(1 + \eta) \quad (3)$$

where η is the steady-state NOE (Noggle & Schirmer, 1971).

(b) *Inversion of S*:

$$I_z(t) = I_0[1 + e^{-(\rho-\sigma)t} - e^{-(\rho+\sigma)t}] \quad (4)$$

In addition, it can be seen that the value of η depends on τ_c. Specifically, from the definition of η in eq 3, we have

$$\eta = \sigma/\rho = \frac{6J(2\omega) - J(0)}{J(0) + 3J(\omega) + 6J(2\omega)} \quad (5)$$

where $J(\omega) = \tau_c/[1 + (\omega\tau_c)^2]$.

† This work was supported by the National Institutes of Health from NIDDK Grants DK43146 and DK27121 to R.G.S. and Biomedical Research Support Grant RR05358 to M.J.A.

* Address correspondence to this author at the Yale University School of Medicine, 126 Magnetic Resonance Center (MRC), 333 Cedar St., P.O. Box 3333, New Haven, CT 06510. Telephone: (203) 785-6622. Fax: (203) 785-6643.

‡ Department of Molecular Biophysics and Biochemistry.

§ Department of Internal Medicine.

® Abstract published in *Advance ACS Abstracts,* October 1, 1993.

0006-2960/93/0432-11483$04.00/0

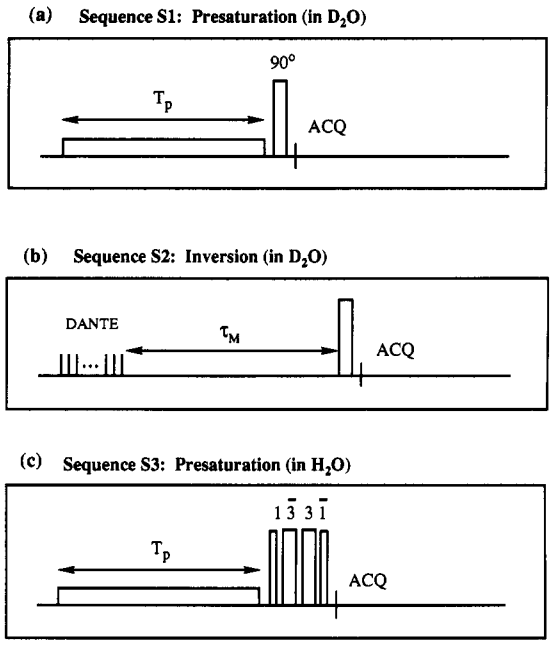

FIGURE 1: Pulse sequences used for generating (a) steady-state NOE in D_2O, (b) transient NOE in D_2O, and (c) steady-state NOE in H_2O. The 1-3-3-1 semiselective pulse was used for water suppression.

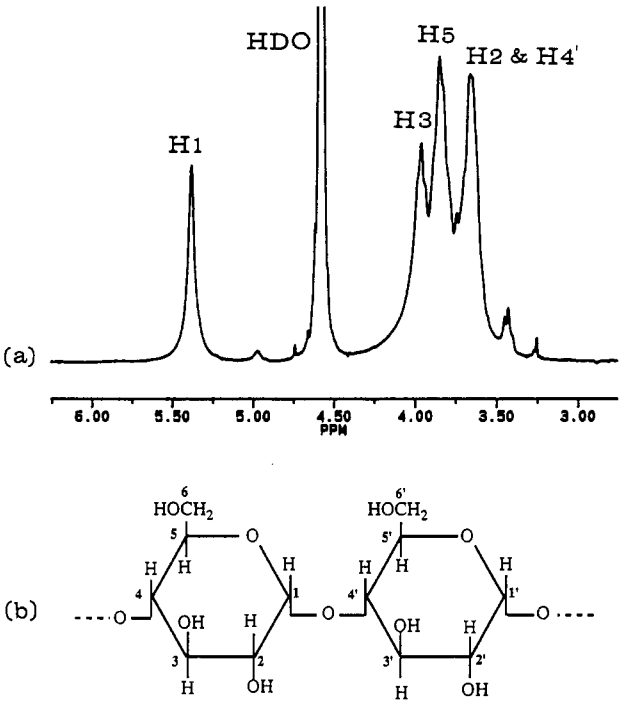

FIGURE 2: (a) ^1H spectrum of glycogen in D_2O collected using a single-pulse acquire sequence at 360 MHz. (b) Structure of two glucose monomer subunits in α-1,4 linkage showing proton numbers.

METHODS

Sample Preparation. Glycogen from the same batch of commercially available glycogen (oyster glycogen type II, Sigma Catalog no. G-8751) was dissolved in D_2O or H_2O to 370 mM. The solutions were adjusted to pH ~7 by adding NaOH.

NMR Methods. ^1H spectra were collected using a Bruker AM 360 (8.4 T) equipped with a 5-mm ^1H probe. Steady-state and transient NOEs were determined using low-power CW irradiation for frequency-selective presaturation sequences (Figure 1a) and DANTE trains (Morris & Freeman, 1978) for frequency-selective inversion sequences (Figure 1b). A 6-s low-power radio-frequency pulse (γB_1 = 7 Hz) was used for presaturation in measurements of steady-state NOEs. The DANTE trains consisted of 70–80 pulses spaced 100–300 μs apart to give selective proton inversion with a negligible direct effect on water. The selective inversion had no direct effect on the other resonances. Water suppression, where necessary, was achieved using a semiselective pulse (Hore, 1983) (see Figure 1c). All spectra were run fully relaxed (at repetition time TR = 15 s for glycogen in D_2O and 10 s for glycogen in H_2O). The 90° pulse width was 8–9 μs. NOEs were measured from the difference between a spectrum collected with presaturation or inversion of the dipolar resonances and a control spectrum collected with the same presaturation or inversion placed symmetrically about the peak whose NOE was under study. All measurements were performed at 37 °C.

RESULTS

Figure 2a shows the ^1H spectrum of glycogen in D_2O at 360 MHz and 37 °C obtained following a nonselective $\pi/2$ pulse. The resonance assignments follow Zang et al. (1991). Figure 2b shows the structure of two glucose monomer subunits in α-1,4 linkage with the conventional proton numbering. The H1 proton with α-1,4 linkage gives rise to the downfield resonance at 5.38 ppm with a line width of ~15 Hz. We measured the steady-state NOE of glycogen H1 following presaturation of H2 and H4′ using sequence S1 (Figure 1a).

Figure 3a is the control glycogen proton spectrum in D_2O with 6 s of presaturation at 7.12 ppm (+626 Hz from H1). Figure 3b shows the spectrum after 6 s of presaturation of the H2 and H4′ resonances at 3.64 ppm (–626 Hz from H1). The difference spectrum (Figure 3c) gave a 74% loss of glycogen H1 intensity (η = –0.74).

The time evolution of the transient NOE in H1 following inversion of H2+H4′ was measured using sequence S2 (Figure 1b). The inversion delay time (τ_m) between selective inversion and detection was varied and the NOE determined from the difference between the transient NOE spectrum and the control spectrum at $\tau_m > 5T_1$. The results are shown in Figure 4. The square symbols are experimental data points, and the solid line is the best fit to eq 1. The glycogen H1 intensity decreased at short τ_m and then slowly recovered to equilibrium. The τ_m at which the measured NOE was largest was 0.11 s; however, there is some uncertainty in the correct value of τ_m at which the peak NOE occurs, due to uncertainties in base-line correction, and relatively low sensitivity in the NOE spectra. Assuming an uncertainty of 5% in NOE intensities, the NOE maximum occurs when τ_m lies between 0.08 and 0.18 s.

Selective inversion of H1 using sequence S2 gave transient NOEs in H3, H5, and H2+H4′. Figure 5 shows the time evolution with τ_m and the relative sizes of these NOEs. The initial rate of appearance of the NOE in the H2+H4′ resonance was ~3.7 times as rapid as the rate of NOE appearance in H3 and H5. The maximum reductions in intensity occurred at $\tau_m \approx 0.15$ s for H2+H4′, $\tau_m \approx 0.30$ s for H3, and $\tau_m \approx 0.45$ s for H5.

We next examined the effect of replacement of D_2O with H_2O on ^1H NOEs in glycogen. Figure 6 shows the dependence of glycogen H1 intensity on the duration (T_p) of presaturation of H2+H4′ in H_2O. The same presaturation field (γB_1 = 7 Hz) was used. The H1 peak decreased with increasing T_p, reaching a steady-state intensity which was 41% lower than control at $T_p \approx 0.8$ s. The steady-state H1 NOE following H2+H4′ saturation was thus lower (η = –0.41) in H_2O than in D_2O (η = –0.74).

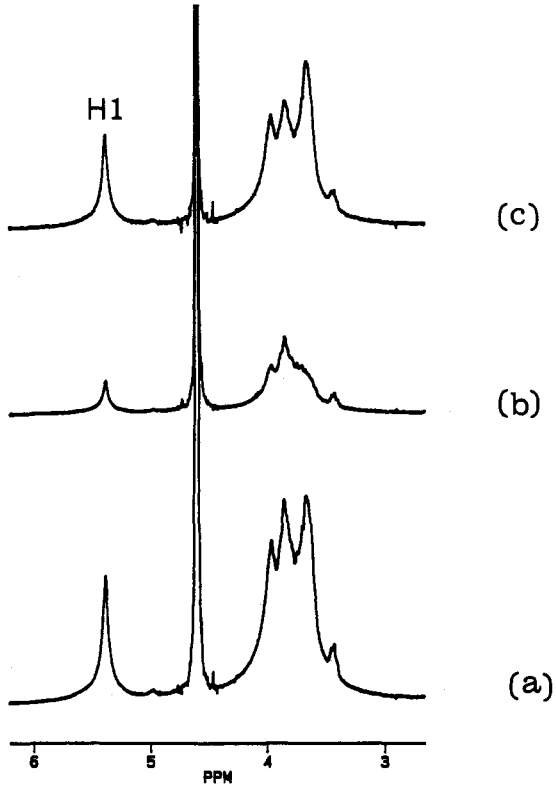

FIGURE 3: (a) Control ^1H spectrum of glycogen in D_2O obtained using pulse sequence S1 with 6-s presaturation 626 Hz downfield of glycogen H1. (b) Spectrum acquired with 6 s of H2 presaturation (626 Hz upfield of H1). (c) Difference spectrum (a–b) showing 74% loss of H1 intensity.

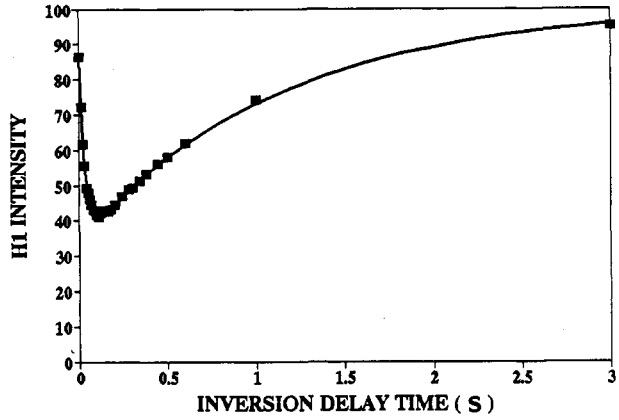

FIGURE 4: Dependence of H1 intensity in D_2O as a function of the inversion delay time τ_m following selective H2 and H4′ inversion. The maximal reduction of H1 intensity appears at $\tau_m \approx 0.11$ s. The data were best fitted to eq 1.

Saturation of H_2O also gave a negative NOE in glycogen H1 (Figure 7). Figure 7a is the control H1 resonance peak in H_2O obtained following 6 s of CW presaturation downfield (+245 Hz) from H1. This peak has the same intensity as the peak obtained following 6 s of presaturation +50 kHz from H1 (Figure 7c). Thus, there was no direct offset presaturation or significant magnetization-transfer effects on the H1 resonance. Figure 7b shows the H1 resonance after 6 s of H_2O presaturation (–245 Hz from H1). The H1 intensity suffered a 55% reduction compared with the H1 peak in Figure 7a. All spectra in Figure 7 were collected using sequence S3 (Figure 1c).

The results of steady-state and transient NOE generations of glycogen in D_2O and H_2O are summarized in Table I.

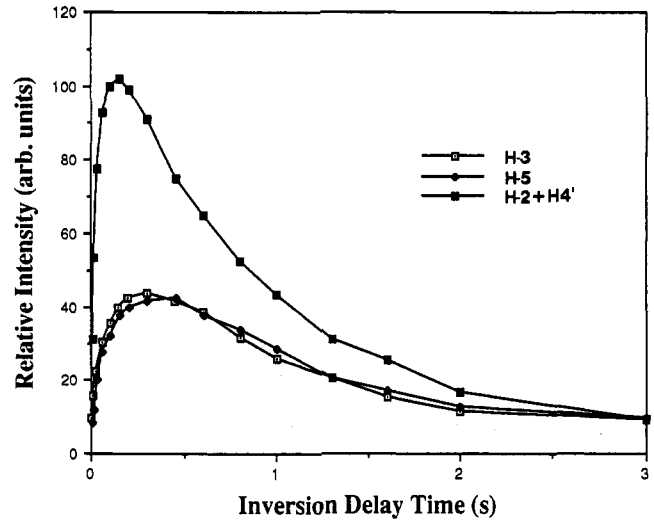

FIGURE 5: H2+H4′, H3, and H5 NOE intensities in D_2O as a function of τ_m, following selective inversion of H1 using the sequence S2. All NOE intensities are negative.

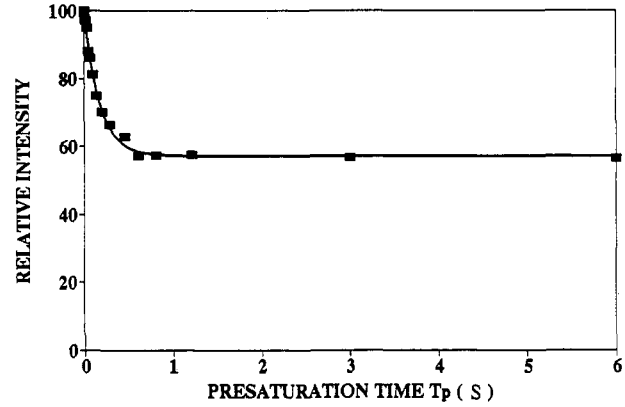

FIGURE 6: Dependence of H1 intensity in H_2O on the duration T_p of H2 and H4′ presaturation using the sequence S3. The data were fitted to eq 2 as described by the solid line.

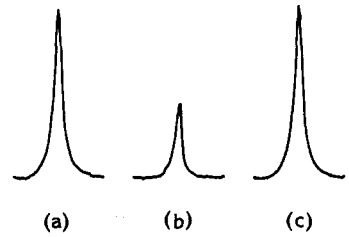

FIGURE 7: (a) H1 resonance of glycogen in H_2O obtained following 6 s of CW irradiation +245 Hz from H1. (b) H1 resonance following 6 s of presaturation of the H_2O peak (–245 Hz from H1). The H1 intensity suffered a 55% reduction compared with (a). (c) H1 resonance following 6 s of presaturation +50 kHz from the H1 position. There was no significant difference in H1 intensity in (c) compared with (a). All spectra were collected using sequence S3.

Table I: Glycogen H1 NOEs

NOE generation	H1 NOE η in D_2O	H1 NOE η in H_2O
saturation of H2 + H4′	–0.74	–0.41
saturation of H_2O		–0.55
inversion of H2 + H4′	–0.60	

DISCUSSION

We showed previously that the relaxation rates of the glycogen H1 proton are dominated by the dipole–dipole interactions between intraring H1–H2 and interring H1–H4′

with an average isotropic τ_c = 2.7 ns (Chen et al., 1993). At 360 MHz, where $\omega\tau_c > 1$ and $W_0 > W_2$, we expect that saturation or inversion of the appropriate dipolar-coupled protons should give a negative NOE. Indeed, with τ_c = 2.7 ns, eq 5 predicts $\eta = -0.86$ for glycogen H1 in D_2O for complete saturation of *all* coupled spins. The experimentally determined NOE was smaller ($\eta = -0.74$) as shown in Figure 3. The calculated η assumes that all the spins coupled to H1 are completely saturated. It is difficult to meet this requirement since it was difficult to fully saturate the H2+H4′, H3, and H5 resonances without partial saturation of H1. Figure 3b shows evidence of incomplete saturation, since a residual signal can be seen in the 3.4–3.9 ppm region corresponding to H3 and H5. Incomplete saturation of H3 and H5 will reduce the size of the NOE observed. Consistent with this, increasing the saturating field to completely saturate the H2+H4′, H3, and H5 resonances gave $\eta \approx -0.85$, relative to the amplitude of the partially saturated control H1 intensity measured in the presence of the same saturating field positioned downfield of H1 by the same frequency offset.

The maximum transient NOE obtained following frequency-selective inversion of the H2 and H4′ resonances was –0.60 (Table I). The minimum η value expected can be estimated using eq 4. It is straightforward to calculate the time (t_{min}) after complete inversion of all coupled spins at which the H1 intensity reaches the lowest value:

$$t_{min} = -1/2\sigma \ln[(\rho-\sigma)/(\rho+\sigma)] \quad (6)$$

Using ρ = 5.4 ± 0.4 s^{-1} and σ = –4.5 ± 0.4 s^{-1} for glycogen H1 in D_2O (Chen et al., 1993), we obtain t_{min} = 0.27 s. The maximal H1 signal loss predicted from eq 4 is

$$[(I_z(t) - I_0)/I_0]_{min} = e^{-(\rho-\sigma)t_{min}} - e^{-(\rho+\sigma)t_{min}} = -0.72 \quad (7)$$

i.e., a 72% H1 signal loss. The observed maximum loss of 60% was smaller than predicted. The experimental t_{min} lies in the range 0.08–0.18 s. This is less than the value predicted assuming complete inversion of all dipolar-coupled spins (0.27 s). We believe this arises from two effects: the incomplete inversion of the H2+H4′ protons and the significant cross-relaxation which occurs during the selective inversion. Assuming ρ = 5.4 s^{-1} for H1, H2, and H4, $\sigma_{12} = \sigma_{14} = -2.3$ s^{-1}, and $\sigma_{24} = 0$, simulation of the time course for the H1 NOE using $M_z = -0.3M_0$ for H2+H4′ and an M_z of $0.8M_0$ for H1 at the end of the selective inversion pulse obtained from experimental results gives a rather flat region from τ_m = 0.12 s to τ_m = 0.27 s during which the NOE is within 5% of its maximum. This is in reasonable agreement with the experimental data.

In addition, the error analysis of eq 6 gives the maximum relative error of t_{min} as described in eq 8:

$$\Delta t_{min}/t_{min} = \frac{(\Delta\rho - \Delta\sigma)/(\rho - \sigma) + (\Delta\rho + \Delta\sigma)/(\rho + \sigma)}{\ln[(\rho - \sigma)/(\rho + \sigma)]} + |\Delta\sigma/\sigma| \quad (8)$$

Using values of ρ = 5.4 s^{-1}, $\Delta\rho$ = 0.4 s^{-1}, σ = –4.5 s^{-1}, and $\Delta\sigma$ = 0.4 s^{-1} shows that $\Delta t_{min}/t_{min}$ = 0.46 and t_{min} = 0.27 ± 0.12 s. This demonstrates a wide range of t_{min} values calculated by eq 6.

The long τ_c and consequent dominance by W_0 of cross-relaxation in glycogen are expected to lead to rapid second-order NOEs. The sizes and rates of appearance of the transient NOEs generated in H3 and H5 relative to H2+H4′ following H1 inversion (Figure 5) are larger than would be expected for first-order NOEs since the H1–H3 and H1–H5 distances are *much* greater than the H1–H2 and H1–H4′ distances (Chen et al., 1993). For direct interactions, the rate of appearance of first-order NOEs has a $1/r^6$ dependence. Thus, if the NOEs in H3 and H5 were due to direct interactions with H1, their rates of appearance should be only ~1% of that for H2+H4′, since $r_{13}^{-6}/(r_{12}^{-6} + r_{14'}^{-6}) \approx 0.008$ and $r_{15}^{-6}/(r_{12}^{-6} + r_{14'}^{-6}) \approx 0.01$ (Chen et al., 1993). In contrast, we observe rates of appearance of NOEs in H3 and H5 which are significantly larger. These rates are consistent with the strong second-order NOEs predicted from the long τ_c and $\omega\tau_c > 1$. Likely paths are H1 → H2 → H3 and H1 → H4′ → H5′.

When H_2O rather than D_2O is the solvent, the effects of direct cross-relaxation with H_2O, cross-relaxation with glycogen OH protons, and chemical exchange of the glycogen OH protons with H_2O must be considered in the analysis of NOEs. Our earlier study (Chen et al., 1993) suggested that there is no significant direct cross-relaxation between H1 and H_2O (since τ_c is too long), and between H1 and glycogen hydroxyl protons (since the distance is too great). However, the additional strong cross-relaxation of H2 and H4′ by glycogen OH protons should lead to significant effects on the H1 relaxation and NOE due to the very efficient H1–H2 and H1–H4′ cross-relaxation pathways. That presaturation of H_2O leads to a large negative steady-state NOE and a 55% signal loss for glycogen H1 confirms that the direct interaction of H1 with H_2O is weak since this should have $\omega\tau_c \ll 1$ and a positive NOE. In fact, the negative NOE indicates that a long τ_c pathway for cross-relaxation of the H_2O pool with H1 dominates. A possible mechanism is by first-order OH–H1 NOEs and/or second-order NOEs such as OH2 ↔ H2 ↔ H1. This H_2O interaction competes with other glycogen spins coupled to H1 and reduces the NOE factor generated by presaturating H2 and H4′ resonances from $\eta = -0.74$ in D_2O to $\eta = -0.41$ in H_2O.

The data from the dynamic presaturation experiment in H_2O were used to fit eq 2. The plot is shown in Figure 6. The best-fit values were $\sigma/\rho = \eta = -0.43 \pm 0.02$ and ρ = 5.8 ± 0.1 s^{-1}, and thus $\sigma = -2.5 \pm 0.2$ s^{-1}. The 95% confidence limits of the values of η and ρ obtained from the fits were determined using a Monte-Carlo estimation based on the noise amplitude in the spectra and the estimated error in base-line correction (Press et al., 1989). This η value is not significantly different from the value of –0.41 obtained from the steady-state presaturation experiment (Table I). The value of ρ is also similar to that obtained from glycogen in D_2O using relaxation analysis (Chen et al., 1993). This again supports the hypothesis that there is no direct cross-relaxation between H_2O or glycogen OH protons and glycogen H1. However, the absolute value of σ is less than the σ value of –4.5 ± 0.4 s^{-1} measured in D_2O. The difference between these cross-relaxation terms must be due to the presence of H_2O and glycogen OH protons. Using the values of σ and ρ determined from the time dependence of the build-up of the steady-state NOE following H2+H4′ presaturation, we predict a $T_{1eff} = (\rho+\sigma)^{-1} = 0.30 \pm 0.02$ s. T_{1eff} is the T_1 for H1 following inversion of H1 and all other dipolar-coupled protons in H_2O. We have previously obtained a value of 0.31 ± 0.01 s for this T_1 using semiselective inversion of H1 and most of the upfield envelope of glycogen proton resonances (Chen et al., 1993), in excellent agreement with the calculated value.

As expected from T_1 data (Chen et al., 1993), the dominant dipolar interactions of H1 are with H4′ and H2. The H4′ and H2 resonances are not well resolved in the ^1H spectrum of glycogen at 360 MHz. However, there is unlikely to be

significant direct interaction between H4′ and H2, due to the significantly longer internuclear distance. These two facts suggested that we could analyze the NOEs of the H1 in terms of a simple two-spin model in which H4′+H2 are treated as a single dipolar interaction. This two-spin treatment gives good quantitative agreement with the experimentally observed NOEs, and has the benefit of providing a simpler physical description of the principal features of cross-relaxation in glycogen. Full relaxation matrix analysis of 2D NOESY and ROESY experiments will be necessary to completely describe the second-order effects which are undoubtedly present. In the absence of the large amounts of 2D data necessary to measure the second-order effects accurately, the number of assumptions necessary to perform a multispin analysis on the 1D data presented herein would effectively reduce it to a two-spin problem. Furthermore, at 360 MHz, with the T_2-dominated line widths, it would be difficult to resolve all of the necessary cross-peaks in the NOESY spectrum. We plan to investigate this at 500 MHz.

CONCLUSIONS

Saturation of the H2+H4′ resonances of glycogen in D_2O gave a 74% reduction of glycogen H1. Selective inversion of the H2+H4′ region followed by an appropriate delay for cross-relaxation to occur gave a 60% maximum reduction in H1 intensity around 0.08–0.18 s after inversion. These observations are consistent with a model in which there are strong H1–H2 and H1–H4′ dipolar interactions. The long τ_c of the dipolar interaction in glycogen generates a large and negative NOE at 360 MHz. The solvent H_2O has a significant contribution to glycogen H1 relaxation with a slow correlation time suggesting an indirect pathway. Saturation of the H_2O resonance gave a 55% reduction in glycogen H1 intensity.

The strong NOEs seen between the ring protons of glycogen suggest a means of editing the glycogen H1 resonance from overlapping resonances *in vivo*. This should permit the measurement of glycogen *in vivo* with high sensitivity using ^1H NMR.

REFERENCES

Chen, W., Zhu, X.-H., Avison, M. J., & Shulman, R. G. (1993) *Biochemistry* (in press).

Gunja-Smith, Z., Narshall, J. J., Mercier, C., Smith, E. E., & Wheelan, W. J. (1970) *FEBS Lett. 12*, 101–104.

Hore, P. J. (1983) *J. Magn. Reson. 55*, 283–300.

Morris, G. A., & Freeman, R. (1978) *J. Magn. Reson. 29*, 433–462.

Noggle, J. H., & Schirmer, R. E. (1971) in *The Nuclear Overhauser Effect. Chemical Applications*, Academic Press, New York.

Press, W. H., Flannery, B. P., Teukolsky, S. A., & Vetterling, W. T. (1989) in *Numerical recipes in Pascal*, pp 572–590, Cambridge University Press, New York.

Sillerud, L. O., & Shulman, R. G. (1983) *Biochemistry 22*, 1087–1094.

Zang, L.-H., Rothman, D. L., & Shulman, R. G. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1678–1680.

Zang, L.-H., Laughlin, M. R., Rothman, D. L., & Shulman, R. G. (1990b) *Biochemistry 29*, 6815–6820.

Zang, L.-H., Howseman, A. M., & Shulman, R. G. (1991) *Carbohydr. Res. 220*, 1–9.